Chapter 11
Structural Biology of Replication Initiation Factor Mcm10

Wenyue Du, Melissa E. Stauffer, and Brandt F. Eichman

Abstract Minichromosome maintenance protein 10 (Mcm10) is a non-enzymatic replication factor required for proper assembly of the eukaryotic replication fork. Mcm10 interacts with single-stranded and double-stranded DNA, DNA polymerase α and Mcm2-7, and is important for activation of the pre-replicative complex and recruitment of subsequent proteins to the origin at the onset of S-phase. In addition, Mcm10 has recently been implicated in coordination of helicase and polymerase activities during replication fork progression. The nature of Mcm10's involvement in these activities, whether direct or indirect, remains unknown. However, recent biochemical and structural characterization of Mcm10 from multiple organisms has provided insights into how Mcm10 utilizes a modular architecture to act as a replisome scaffold, which helps to define possible roles in origin DNA melting, Pol α recruitment and coordination of enzymatic activities during elongation.

Keywords DNA binding • DNA replication • Mcm10 • Minichromosome maintenance • Protein-protein interaction

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11.1 Replication Initiation

DNA replication can be divided into three primary stages: initiation, elongation and termination (Bell and Dutta 2002; Garg et al. 2005). Initiation commences during the G1-phase of the cell cycle, during which the replisome – the protein complex responsible for DNA unwinding and synthesis at an active replication fork – begins to assemble at origins of replication (Fig. 11.1). Initiation begins with origin licensing, in which the origin recognition complex (ORC), coupled with Cdc6 and Cdt1, loads the minichromosome maintenance (Mcm) proteins Mcm2-7 onto DNA as a head-to-head double hexamer (Remus et al. 2009). This marks the formation of the pre-replicative complex (pre-RC), which remains inactive in G1-phase.

The transition to S-phase is accompanied by origin activation. Mcm10 is one of the first proteins loaded onto chromatin at the onset of S-phase and it is essential for the subsequent recruitment of other replisome proteins (Wohlschlegel et al. 2002). At this point, two phosphorylation events take place to activate the pre-RC. In yeast, cyclin-dependent kinase (CDK) phosphorylates Sld2 and Sld3 and facilitates their binding to Dpb11 (Tanaka et al. 2007; Zegerman and Diffley 2007) and Dbf4-dependent kinase (DDK), composed of Cdc7 and Dbf4, directly phosphorylates Mcm2 and Mcm4 (Lei et al. 1997; Sheu and Stillman 2006). Mcm10 is important for both of these events. It has been shown to stimulate Mcm2-7 phosphorylation by DDK and may, in fact, recruit DDK to the pre-RC (Lee et al. 2003). In addition, the human RecQ4 helicase contains a Sld2-like sequence that is both a phosphorylation target of CDK and a binding site for Mcm10, suggesting that phosphorylation may act as a switch for RecQ4 activity by modulating its interaction with Mcm10 (Xu et al. 2009). These phosphorylation events enable the subsequent loading of two helicase cofactors, Cdc45 and the GINS complex, to form the pre-initiation complex (pre-IC) with the help of Mcm10 and other factors, including And-1/Ctf4 (Im et al. 2009; Tanaka and Nasmyth 1998; Wohlschlegel et al. 2002; Zou and Stillman 2000). Cdc45, GINS, and Mcm2-7 form the CMG complex, which is considered to be the active form of the replicative helicase (see Chaps. 6–8, this volume; Costa et al. 2011; Gambus et al. 2006; Ilves et al. 2010; Moyer et al. 2006; Pacek et al. 2006). Denaturation of origin DNA into single strands forms two bidirectional replication forks and is marked by recruitment of single-stranded DNA (ssDNA) binding protein replication protein A (RPA, see Chap. 10, this volume).

The initiation phase concludes upon recruitment of the DNA synthesis machinery to the emerging replication fork. Fork firing requires DNA polymerase α (Pol α)-primase to initiate DNA synthesis by generating RNA primers and short stretches of DNA on both leading and lagging strands (see Chap. 9, this volume). Mcm10 and And-1/Ctf4 have been implicated in loading Pol α onto chromatin, as well as physical coupling of Pol α and Mcm2-7 (Gambus et al. 2009; Im et al. 2009; Lee et al. 2010; Ricke and Bielinsky 2004; Zhu et al. 2007). Elongation proceeds through processive DNA synthesis by replicative DNA polymerases δ and ε, which require the sliding clamp, proliferating cell nuclear antigen (PCNA), and the clamp loader, replication factor C (RFC) (see Chaps. 12–15, this volume). Fork progression requires concerted DNA unwinding and synthesis through coordination of activities among the CMG complex and polymerases α, δ, and ε.

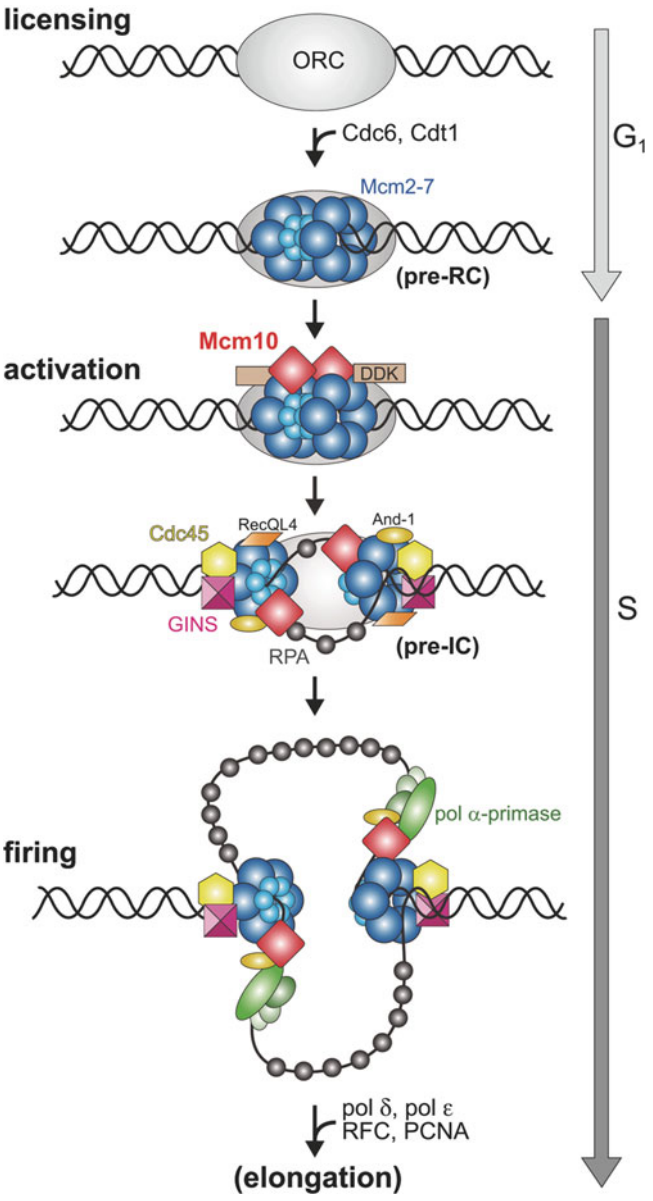


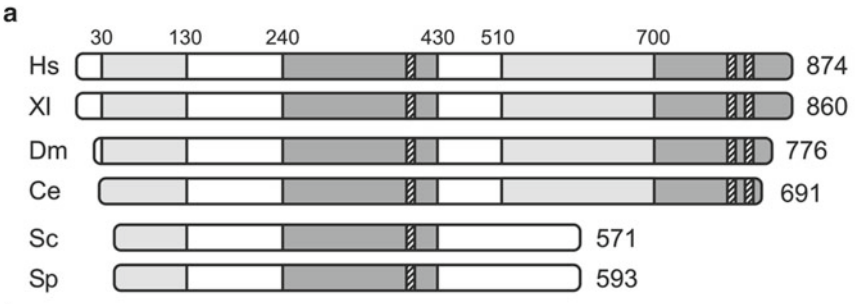
Fig. 11.1 A simplified view of the initiation phase of eukaryotic replication, highlighting key steps involved in replisome assembly. Many replication factors are omitted for clarity. At the end of the G1 phase of the cell cycle, chromatin is licensed for replication at the origin by formation of a pre-replicative complex (pre-RC), which includes an inactive Mcm2-7 helicase. At the onset of S-phase, the pre-RC is activated by Dbf4-dependent kinase (DDK) phosphorylation. Mcm10 loads in early S-phase and is required for loading of Cdc45 and GINS, which form the CMG helicase complex with Mcm2-7 and help constitute a pre-initiation complex (pre-IC). Denaturation of origin DNA allows for binding of DNA polymerases and the rest of the elongation machinery, stimulating origin firing. Mcm10 and And-1/Ctf4 have been implicated in coupling Pol α to the replisome

11.2 Role of Mcm10 in Replication

The gene encoding Mcm10 was first identified in genetic screens in yeast. Referred to at the time as Cdc23, Mcm10 was shown to be necessary for cell division in *Schizosaccharomyces pombe* (Aves et al. 1998; Nasmyth and Nurse 1981). Bulk DNA synthesis was disrupted in temperature sensitive alleles of *cdc23*, and thus DNA replication and mitosis were blocked. Similar genes, referred to as *DNA43* and *MCM10*, were identified in screens in *Saccharomyces cerevisiae* and shown to encode homologs of Cdc23 (Dumas et al. 1982; Maine et al. 1984). *DNA43* was found to be essential for entering S-phase and maintaining cell viability (Solomon et al. 1992). Ricke and Bielinsky (2004) showed that the recruitment of *S. cerevisiae* Mcm10 (scMcm10) to replication origins is cell cycle regulated and dependent on pre-RC assembly, and that scMcm10 is required to maintain Pol α on chromatin independently of Cdc45. The importance of Mcm10 to replication initiation in yeast is evident from the number of genetic and physical interactions identified between Mcm10 and proteins involved in origin recognition, replisome assembly, and fork progression (Gregan et al. 2003; Hart et al. 2002; Homesley et al. 2000; Kawasaki et al. 2000; Merchant et al. 1997; Sawyer et al. 2004).

Mcm10 homologs have also been identified and characterized in higher eukaryotes, including humans, *Xenopus* and *Drosophila* (Christensen and Tye 2003; Izumi et al. 2000; Wohlschlegel et al. 2002). Human Mcm10 (hMcm10) interacts with chromatin at the G1/S-phase transition and dissociates in G2-phase (Izumi et al. 2000). It is important for activation of pre-RCs and functional assembly of the replisome and is regulated by phosphorylation-dependent proteolysis during late M- and early G1-phase (Izumi et al. 2001). Studies in *Xenopus* extracts showed that Mcm10 (xMcm10) binds to the pre-RC at the onset of S-phase, with roughly one xMcm10 bound per 5,000 bp of DNA (approximately two Mcm10s per active origin) (Wohlschlegel et al. 2002). These studies also showed Mcm10 to be essential for loading downstream proteins Cdc45 and RPA (Wohlschlegel et al. 2002), which are in turn required for chromatin unwinding and the association of Pol α at the origin (Walter and Newport 2000). The *Drosophila* homolog of Mcm10 was able to complement an *mcm10*-null strain of *S. cerevisiae* and was shown to interact with many members of the pre-RC in KC cells, including Mcm2, ORC and Cdc45 (Christensen and Tye 2003). Depletion of Mcm10 from KC cells led to defects in chromosome condensation (Christensen and Tye 2003).

The human, *Xenopus* and *Drosophila* Mcm10 orthologs have high sequence similarity but are distinct from the yeast proteins in several ways. First, the vertebrate proteins have an additional C-terminal domain (Robertson et al. 2008) (Fig. 11.2a). Second, phosphorylated and mono- and diubiquitylated forms of hMcm10 have been identified (Izumi et al. 2001), whereas only diubiquitylated Mcm10 has been shown to be associated with chromatin in yeast (Das-Bradoo et al. 2006). Finally, *S. pombe* Mcm10/Cdc23 (spMcm10) has been reported to contain primase activity (Fien and Hurwitz 2006), a function not observed in other orthologs.



b

species	Oligomeric state	Method used	Reference
S.pombe	Monomer (asymmetric) Dimer	GFC, GGS GFC, GGS	Lee et al. 2003 Fien and Hurwitz 2006
S.cerevisiae	Dodecamer or larger Monomer (dsDNA), Trimer (ssDNA)	GFC, GGS EMSA, SPR	Cook et al. 2003 Eisenberg et al. 2009
X. laevis	FL, Dimer or larger (asymmetric) NTD, Dimer (asymmetric)	AUC	Robertson et al. 2008
H. sapiens	Hexamer	AUC, EM	Okorokov et al. 2007

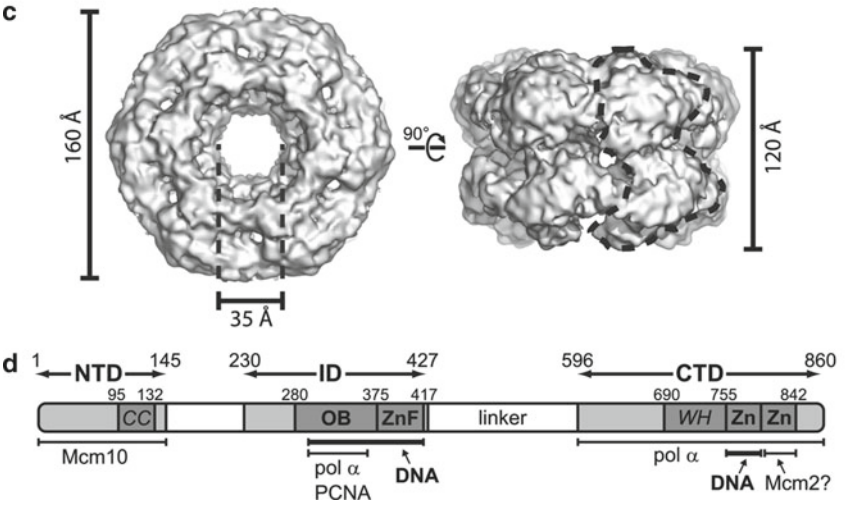


Fig. 11.2 Mcm10 sequence homology, oligomerization, and domain architecture. (**a**) A schematic sequence alignment of Mcm10 from *Homo sapiens (Hs), Xenopus laevis (Xl), Drosophila melanogaster (Dm), Caenorhabditis elegans (Ce), Saccharomyces cerevisiae (Sc),* and *Schizosaccharomyces pombe (Sp). Light* and *dark grey bars* indicate regions of moderate and high sequence conservation, respectively, and *hatched boxes* represent invariant cysteine/histidine clusters involved in zinc coordination. (**b**) Various oligomeric states of Mcm10 reported in the literature. *GFC* gel filtration chromatography; *GGS* glycerol gradient sedimentation; *EMSA* electrophoretic mobility shift assay, *SPR* surface plasmon resonance, *AUC* analytical ultracentrifugation, *EM* electron microscopy. (**c**) Orthogonal views of an EM reconstruction of human Mcm10 at 16 Å resolution and contoured at 1σ. The *dashed line* represents one 95-kDa subunit. (**d**) Domain architecture of *Xenopus laevis* Mcm10. *NTD* N-terminal domain, *ID* internal domain, *CTD* C-terminal domain, *CC* predicted coiled coil, *OB* oligonucleotide/oligosaccharide binding fold, *ZnF* zinc-finger, *WH* predicted winged helix, *Zn* zinc ribbon. Interactions with proteins and DNA are shown below the schematic

Physical interactions have been observed between Mcm10 and multiple proteins found in the pre-RC and at the replication fork, including ORC (Hart et al. 2002; Izumi et al. 2000), Mcm2-7 (see below), Pol α (Chattopadhyay and Bielinsky 2007; Ricke and Bielinsky 2004, 2006) and the recently identified sister chromatid cohesion protein And-1 and the RecQ-like helicase RecQ4 (Xu et al. 2009; Zhu et al. 2007). spMcm10 interacts with Mcm4/6/7 and Dfp1, the *S. pombe* homolog of Dbf4 (Lee et al. 2003). Furthermore, spMcm10 has been reported to stimulate DDK phosphorylation of Mcm2-7 (Lee et al. 2003) and is thus believed to play a role in helicase activation. Studies in *S. cerevisiae* have shown that scMcm10 facilitates assembly of the Cdc45/Mcm2-7/GINS helicase complex (Gambus et al. 2009) and physical interactions have been observed with Mcm2, Mcm4, Mcm5, Mcm6, and Mcm7 subunits (Apger et al. 2010; Hart et al. 2002; Homesley et al. 2000; Merchant et al. 1997). Recent work suggested that scMcm10 serves as a functional linker between the MCM helicase and Pol α by coordinating their activities and ensuring their physical stability and integrity at the replication fork (Lee et al. 2010), a role also identified for Ctf4 (Gambus et al. 2009). *Drosophila* Mcm10 interacts with Mcm2, Dup (Cdt1), Orc2, Cdc45 and Hp1 in yeast two-hybrid assays (Christensen and Tye 2003). xMcm10 interacts with And-1/Ctf4 (Zhu et al. 2007) and with the helicase/nuclease Dna2 (Wawrousek et al. 2010). hMcm10 interacts with Orc2, Mcm2, and Mcm6 (Izumi et al. 2000) and assembly of the Cdc45/Mcm2-7/GINS complex in human cells requires Mcm10 as well as the And-1/Ctf4 and RecQL4 proteins (Im et al. 2009). hMcm10 also regulates the helicase activity of RecQ4 by direct binding (Xu et al. 2009).

Biochemical studies of Mcm10 have focused on its interactions with DNA and Pol α. Mcm10 binds to both single-stranded (ss) and double-stranded (ds) DNA, with about a three- to five-fold preference for ssDNA (Eisenberg et al. 2009; Fien et al. 2004; Robertson et al. 2008). Fien et al. (2004) showed that spMcm10 can stimulate DNA polymerase activity by interacting with both ssDNA and Pol α, leading to the idea that Mcm10 may facilitate the binding of polymerase complexes to primed DNA. Indeed, Mcm10 affects the localization and stability of Pol α, further supporting the idea that Mcm10 acts as a molecular chaperone for Pol α *in vivo* (Chattopadhyay and Bielinsky 2007; Ricke and Bielinsky 2004, 2006; Yang et al. 2005). Mcm10 interacts with the p180 subunit of Pol α in both yeast and *Xenopus* (Fien et al. 2004; Lee et al. 2010; Robertson et al. 2008). In fact, ssDNA and the N-terminal region of p180 compete for binding to the conserved internal domain of Mcm10 (Warren et al. 2009). Mcm10 can stabilize Pol α throughout the cell cycle by preventing its degradation by the proteasome (Chattopadhyay and Bielinsky 2007; Ricke and Bielinsky 2004, 2006). Moreover, Mcm10 appears to be a cofactor for Pol α activity by increasing its affinity for DNA (Fien et al. 2004; Zhu et al. 2007).

11.3 Overall Architecture

The Mcm10 protein exists only in eukaryotes; no orthologs have been identified in archaea or bacteria, although loose homology has been observed between regions of Mcm10 and the Mcm2-7 proteins (Robertson et al. 2010). Mcm10 proteins range in

size from 571 amino acids in yeast to 874 in humans, with regions of sequence homology clustered in the central and extreme N- and C-terminal regions (Fig. 11.2a). The spacing of homologous regions suggests the presence of three distinct structured domains tethered by unstructured linkers. Zinc finger motifs, initially identified from sequence alignments (Homesley et al. 2000; Izumi et al. 2000) and later confirmed by structural analysis (Robertson et al. 2010; Warren et al. 2008, 2009), are present in both the central and C-terminal regions. The yeast homologs lack the C-terminal region altogether (Robertson et al. 2008), suggesting that in lower organisms, the essential functions of Mcm10 reside within its N-terminal and central regions.

Biochemical and structural studies using vertebrate and yeast Mcm10 orthologs have been rather controversial with regard to the architecture and oligomeric state of the full-length Mcm10 protein (Fig. 11.2b). scMcm10 has been reported to form large, 800 kDa homocomplexes consisting of ~12 molecules when analyzed by size-exclusion chromatography (Cook et al. 2003), although the shape of the molecule could potentially confound this analysis. Self-association in yeast was shown to be mediated by the central zinc finger-containing domain, and mutations in the zinc-binding residues rendered yeast cells temperature-sensitive, with demonstrable replication defects (Cook et al. 2003; Homesley et al. 2000). A more recent surface plasmon resonance study showed that in the presence of ssDNA, scMcm10 forms complexes with three subunits (Eisenberg et al. 2009). On dsDNA, however, scMcm10 interacted as a monomer with a stoichiometry directly proportional to the length of the DNA (~1 scMcm10 per 21–24 bp). Work from the Hurwitz laboratory has reported highly asymmetric monomeric and dimeric forms of spMcm10 using glycerol gradient centrifugation (Fien and Hurwitz 2006; Lee et al. 2003). Analytical ultracentrifugation of xMcm10 was consistent with self-associated, asymmetric complexes, although the precise oligomeric state could not be determined from the data (Robertson et al. 2008). More recent work using size exclusion chromatography with multi-angle light scattering is indicative of xMcm10 complexes containing two to three subunits in the absence of DNA (W. Du and B.F. Eichman, unpublished). This is consistent with the presence of a coiled-coil domain at the N-terminus of the protein (Robertson et al. 2008) and the calculation of two molecules per replication origin based on the concentration of chromatin-bound Mcm10 in *Xenopus* extracts (Wohlschlegel et al. 2002).

hMcm10 was reported to form a ring-shaped hexameric structure using electron microscopy (EM) and single-particle analysis (Okorokov et al. 2007). The particle has dimensions of 160 Å × 120 Å, a 35 Å central channel (Fig. 11.2c) and a system of smaller lateral channels and inner chambers. The volume of the electron density calculated at the 1σ contour level using Chimera (Pettersen et al. 2004) is consistent with a particle of molecular weight 570 kDa or six 95-kDa subunits (unpublished result). From the side, individual subunits appear to adopt two distinct lobes. Model fitting with the structures available at the time suggested that each subunit within the hexamer had the same orientation, with the zinc molecules positioned toward the upper and lower edges of the ring (Okorokov et al. 2007). Subsequent crystal and NMR structures of individual Mcm10 domains, discussed below, cannot be unambiguously positioned into the EM density. The hexameric structure was reportedly consistent with its sedimentation behavior by analytical ultracentrifugation, although the experimental data were not presented (Okorokov et al. 2007).

The authors of the EM structure provided two explanations for hexamerization of Mcm10. The first was that this architecture may enable a topological link with DNA to allow for processive DNA binding like many other ring-shaped DNA-binding proteins. Another explanation was that Mcm10 inherited the hexameric fold from a DNA helicase ancestor but lost the helicase activity during evolution and instead now serves as a "docking" module to facilitate protein-protein interactions in DNA replication, such as Mcm2-7 helicase and Pol α (Chen et al. 2005; Okorokov et al. 2007; Pape et al. 2003; Patel and Picha 2000). It is enticing to speculate that a hexameric Mcm10 structure would provide an extensive binding interface for the six subunits of Mcm2-7, although there are no data to support such a hexamer-hexamer interaction and Mcm10 does not travel with the helicase that has been uncoupled from the replisome by inhibition of the polymerase with aphidicolin (Pacek et al. 2006). In light of the facts that a hexameric form of Mcm10 has not been reported in non-human orthologs, that other studies identify Mcm10 assemblies composed of two to three subunits and that only two molecules of Mcm10 are likely present at the origin, we offer an additional explanation—that the hexamer is simply one of several states occurring in cellular equilibrium and is needed under specific conditions during the onset of replication. For example, hexamerization may be used for sequestering the molecule at the replication fork or as a compact storage state of the protein during replication inactivity. We note that to be consistent with the available oligomerization data, the Mcm10 hexamer may in fact be a trimer of dimers.

11.4 Mcm10 Domain Structure

Biochemical and structural studies have been performed using xMcm10, which has 84% sequence similarity and 58% identity to the human protein. Limited proteolysis and mass spectrometry revealed that full-length xMcm10 is composed of three structured domains at the N-terminal (NTD; residues 1–145), internal (ID; 230–427) and C-terminal (CTD; 596–860) regions of the protein (Fig. 11.2d) (Robertson et al. 2008). The functional significance of the NTD is currently undefined, while the ID and CTD each bind DNA and Pol α (Robertson et al. 2008). Interdomain linkers are predicted to be largely unstructured by secondary structure and disorder predictions and by virtue of their extreme proteolytic sensitivity in purified preparations (Robertson et al. 2008).

11.4.1 Mcm10-NTD

Circular dichroism indicates that the NTD is predominantly α helical and random coil, consistent with secondary structure predictions. The NTD alone is a dimeric assembly as judged by analytical ultracentrifugation, consistent with the presence of a predicted coiled-coil motif comprising residues 93–132 (Robertson et al. 2008).

A strong yeast one-hybrid interaction from the first 100 residues of *Drosophila* Mcm10 was recently reported (Apger et al. 2010), suggesting that the NTD might function as an oligomerization domain for the full-length protein. Interestingly, self-interaction of ID and CTD regions was observed in yeast 2-hybrid assays when the NTD was deleted, suggesting that the NTD may not be the only point of contact between Mcm10 subunits. Nonetheless, the strong NTD self-interaction supports a proposed model in which Mcm10 forms a dimer with two subunits oriented in the same direction, which provides a plausible explanation for interaction of Mcm10 with both leading and lagging strand polymerases at a replication fork. Unlike the ID and CTD, the NTD does not bind to DNA (Robertson et al. 2008).

11.4.2 Mcm10-ID

The ID (residues 230–427) is homologous across all species from vertebrates to yeast and is the most conserved region in the entire protein (Izumi et al. 2000). Mutations in this region were identified in yeast genetic screens to affect minichromosome maintenance and replication *in vivo* (Grallert and Nurse 1997; Liang and Forsburg 2001; Maine et al. 1984; Nasmyth and Nurse 1981). The ID contains a CCCH-type zinc finger and an oligonucleotide/oligosaccharide binding (OB)-fold (Fig. 11.2d) that are now known to facilitate interactions with a number of proteins and DNA (Izumi et al. 2000; Ricke and Bielinsky 2006; Warren et al. 2008). Specifically, the ID has been shown to interact with ssDNA and the N-terminal 323 residues of Pol α (Robertson et al. 2008). In addition, a PCNA interacting peptide (PIP) region was identified in the sequence of scMcm10's ID (Das-Bradoo et al. 2006). Mutations within the PIP box abrogated the interaction between diubiquitylated Mcm10 and PCNA (Das-Bradoo et al. 2006; Warren et al. 2009).

The crystal structure of xMcm10-ID revealed that this region forms a globular domain consisting of an α-helical/random coil region (αA-αB, residues 230–283), an OB-fold (β1-β5.2, residues 286–375) and a C-terminal zinc finger motif (βC-αE, residues 378–418) (Fig. 11.3a). The α-helical/coil region packs onto the back of the OB-fold, opposite the canonical DNA-binding cleft of the OB-fold, to form a flat molecular surface (Warren et al. 2008). The zinc finger protrudes sideways relative to the OB-fold cleft and makes extensive electrostatic and van der Waals contacts with both the L23 loop of the OB-fold and the α-helical/coil region. It is interesting to note that the sequential arrangement of the OB-fold and the zinc finger in Mcm10-ID is different from other DNA processing proteins that contain both structural motifs. In the structures of the archaeal MCM helicase (see Chap. 6, this volume; Fletcher et al. 2003), the RPA trimerization core (see Chap. 10, this volume; Bochkareva et al. 2002), T4 gp32 (Shamoo et al. 1995), and NAD^+-dependent DNA ligase (Lee et al. 2000), a zinc ribbon is inserted into the OB-fold L12 loop, whereas in Mcm10-ID the zinc finger is C-terminal to the entire OB-fold (Warren et al. 2008) (Figs. 11.3a, d). The unique arrangement of the OB-fold/zinc finger suggests that, in Mcm10, this domain assembly may have a unique function.

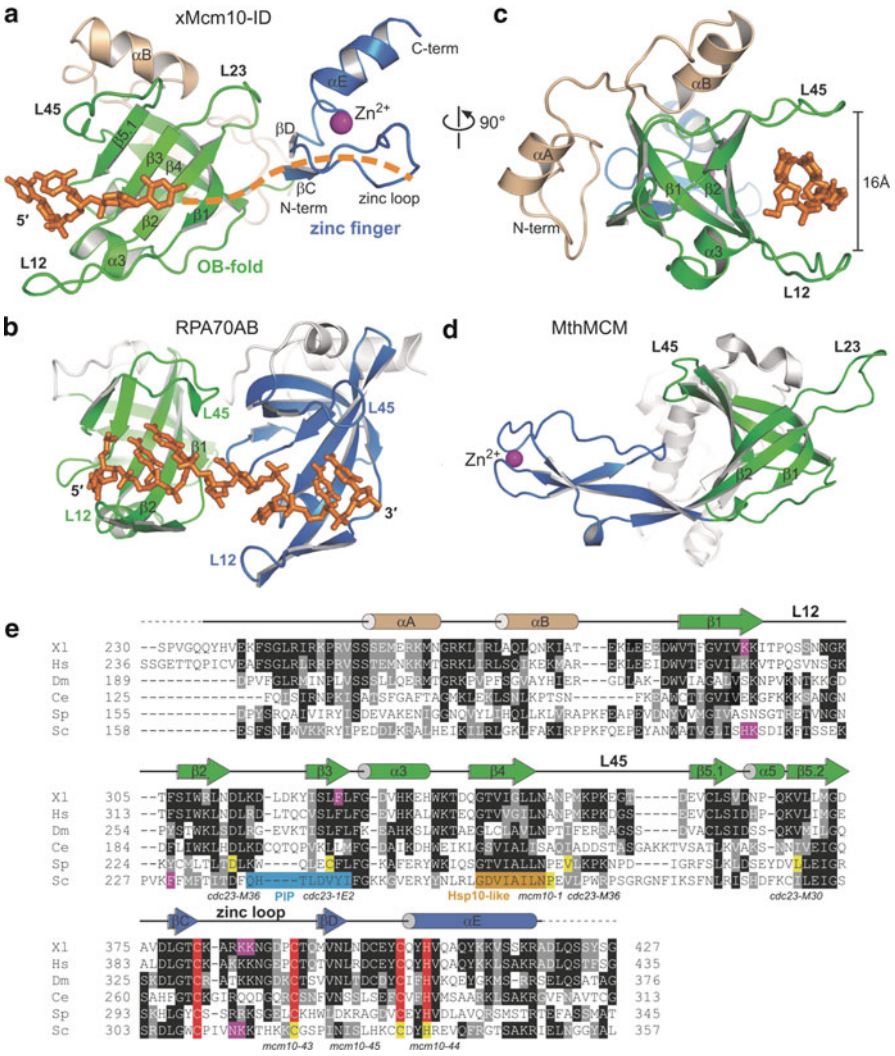


Fig. 11.3 The crystal structure of xMcm10-ID bound to ssDNA. (**a**) xMcm10-ID (residues 235–419) bound to ssDNA, with the OB-fold in *green*, the zinc finger in *blue*, the Zn^{2+} ion in *magenta*, and the N-terminal α-helical/coil region in *tan*. The three nucleotides of ssDNA observed in the crystal structure are shown as *orange sticks*. ssDNA traverses both the OB-fold cleft and the zinc loop. The trajectory of bound DNA determined by NMR is represented by the *orange dashed line*. (**b**) Crystal structure of RPA70AB subunit bound to ssDNA (PDB ID 1JMC). The OB-folds are colored *green* and *blue*, and the ssDNA *orange*. (**c**) Crystal structure of the xMcm10/ssDNA complex viewed 90° with respect to the view shown in panel (**a**). (**d**) Crystal structure of *Methanobacterium thermoautotrophicum* MCM (PDB ID 1LTL). (**e**) Sequence alignment of Mcm10-ID. Zn^{2+}-coordinating residues are highlighted in *red*. Mutations identified in yeast genetic screens to affect cell growth and DNA replication are highlighted in *yellow*. Residues that affect xMcm10 binding to DNA *in vitro* or that increase the sensitivity of *S. cerevisiae* to HU are highlighted in *pink*. The PIP-box and Hsp10-like motif are highlighted in *blue* and *gold*, respectively

The tandem OB-fold-zinc finger arrangement in xMcm10-ID is reminiscent of the high affinity ssDNA binding surface created by side-by-side OB-folds in the RPA70AB sub-domain (Bochkarev et al. 1997) (Fig. 11.3b). NMR chemical shift perturbation of xMcm10-ID indicated that ssDNA binds to both the OB-fold cleft and to the extended loop of the highly basic zinc finger (Warren et al. 2008). The nature of the ssDNA interaction with the concave cleft of the OB-fold was revealed by the crystal structure of xMcm10-ID in complex with ssDNA (Warren et al. 2009). A tricytidine oligonucleotide was clearly observed within the OB-fold cleft, traversing β strands β1- β3 and β5.1 (Fig. 11.3a). The channel created by loops L12 and L45 was ~16 Å in diameter (Fig. 11.3c), allowing the ssDNA a degree of flexibility that precluded observation of atomic-level interactions. However, the polarity of the ssDNA was unmistakably defined, with the 5′ end oriented toward β5.1 and the 3′ end toward β1 and the zinc finger, similar to the polarity reported for the RPA70AB structure (Bochkarev et al. 1997) (Fig. 11.3b). Also similar to RPA70AB, the L12 loop was unobservable in the unliganded structure, presumably due to flexibility (Warren et al. 2008), but upon DNA binding, its electron density was readily visible (Warren et al. 2009).

The Mcm10-ID zinc finger extends the ssDNA binding surface of the OB-fold in a manner analogous to the RPA70B subunit (Bochkarev et al. 1997). A crystal lattice contact occluded DNA binding by the zinc finger in the Mcm10-ID/ssDNA complex structure (Warren et al. 2009). Nonetheless, NMR chemical shift perturbation had unequivocally showed both this region and the cleft between it and the OB-fold to be affected by ssDNA binding, and residues in these regions were shown to affect DNA binding by xMcm10-ID and replication in yeast (Warren et al. 2008). A Lys385Glu/Lys386Glu double mutant on the extended zinc loop reduced ssDNA binding affinity by tenfold, and a Lys293Ala mutant in the cleft reduced it by fivefold (Warren et al. 2008). Transferring these mutations to yeast for assessment of their functional consequences showed that they increased the sensitivity of yeast cells to hydroxyurea (Warren et al. 2008). The Lys293Ala mutation (His215Ala/Lys216Ala in scMcm10) caused a twofold decline in cell survival, while the Lys385Glu/Lys386Glu mutation (Asn313Glu/Lys314Glu in yeast) led to a striking sevenfold decrease. Cell survival was also significantly compromised (~60%) in cells containing the Phe306Ala mutation (Phe230Ala/Phe231Ala in yeast), which resides in the cleft between the OB-fold and zinc finger. Interestingly, the zinc finger domain was also found to be affected by dsDNA binding (Warren et al. 2008). The presence of an extended loop in the zinc finger renders it structurally distinct from the archetypical Zif268 zinc finger that binds dsDNA in a sequence dependent manner, so it remains to be seen how dsDNA binds to this motif.

The Mcm10-ID crystal structures elucidated the yeast mutations originally identified to affect minichromosome maintenance and DNA replication (Fig. 11.3e). The *cdc23-1E2* (Cys239Tyr) (Grallert and Nurse 1997) and *cdc23-M30* (Leu287Pro) (Liang and Forsburg 2001) mutations, which correspond to xMcm10 Leu323 and Leu369, respectively, are located in the interior of the OB-fold's β-barrel, and thus are likely to cause structural perturbations that disrupt protein folding. Other mutations probably affected protein interactions necessary for replisome formation and/or

progression. These include *cdc23-M36* (Asp232Gly), corresponding to the invariant xMcm10 Asp313 that lies on the interior of the L23 loop, and *cdc23-M36* (Val265Ile) and *mcm10-1* (Pro269Leu), which map to solvent exposed positions in the L45 loop (Maine et al. 1984; Nasmyth and Nurse 1981). The human counterpart to the xMcm10-ID has been crystallized (Jung et al. 2008), but the structure was never determined and is expected to be virtually identical to the reported *Xenopus* domain on the basis of high sequence homology (58% identity; 84% similarity).

11.4.3 Mcm10-CTD

Vertebrate homologs of Mcm10 contain a CTD that is unique to higher eukaryotes; yeast Mcm10 is not predicted by sequence alignments to have this domain (Fig. 11.2a). Interactions between xMcm10-CTD (residues 596–860) and ssDNA, dsDNA, and Pol α have been mapped to a proteolytically stable subdomain (residues 690–842) that consists of a putative winged helix motif (residues 690–755) followed by tandem CCCH- and CCCC-type zinc motifs (residues 756–842) (Fig. 11.4a) (Robertson et al. 2010; Robertson et al. 2008). Heteronuclear NOE experiments on this region showed that the putative winged helix contains high backbone flexibility while the zinc motif is more rigid (Robertson et al. 2010). The two Zn^{2+} atoms in xMcm10-CTD, originally identified by atomic absorption spectroscopy, likely play a structural role based on the observations that, in the presence of EDTA, the CTD is more proteolytically sensitive and DNA binding affinity decreases (Robertson et al. 2008).

The solution NMR structure of the zinc binding region of xMcm10-CTD revealed a V-shaped globular domain in which the two zinc binding motifs are tethered by a hinge and the two zinc atoms bind at the tips of the V (Fig. 11.4b) (Robertson et al. 2010). The N-terminal CCCH zinc motif (residues 756–795) consists of a three-stranded antiparallel β-sheet capped with a short perpendicular α-helix with a Zn^{2+} ion embedded in between. DNA binding maps to the CCCH zinc motif, the structure of which is unique to Mcm10. The residues involved in DNA binding trace a nearly continuous 35 Å path around the CCCH arm (Robertson et al. 2010). The length of DNA required for maximal binding affinity was between 10 and 15 nucleotides, suggesting that all of the residues along that path are involved to some extent in interactions with DNA.

The CCCC zinc motif (residues 796–830) adopts a twisted antiparallel β-sheet with the zinc coordinated between the loops by the four cysteines. This motif is not involved in DNA binding and, interestingly, is identical in structure to a zinc ribbon motif in the N-terminal domain of *Methanobacterium thermoautotrophicum* MCM helicase (mtMCM) (Fig. 11.4c, see also Chap. 6, this volume). This MCM zinc motif mediates the head-to-head double hexamer assembly observed in mtMCM crystals (Fletcher et al. 2003, 2005) and in scMcm2-7 loaded onto DNA (Remus et al. 2009). The sequence of the CCCC zinc motif in xMcm10-CTD is highly conserved relative to those in the metazoan Mcm2-7 subunits and

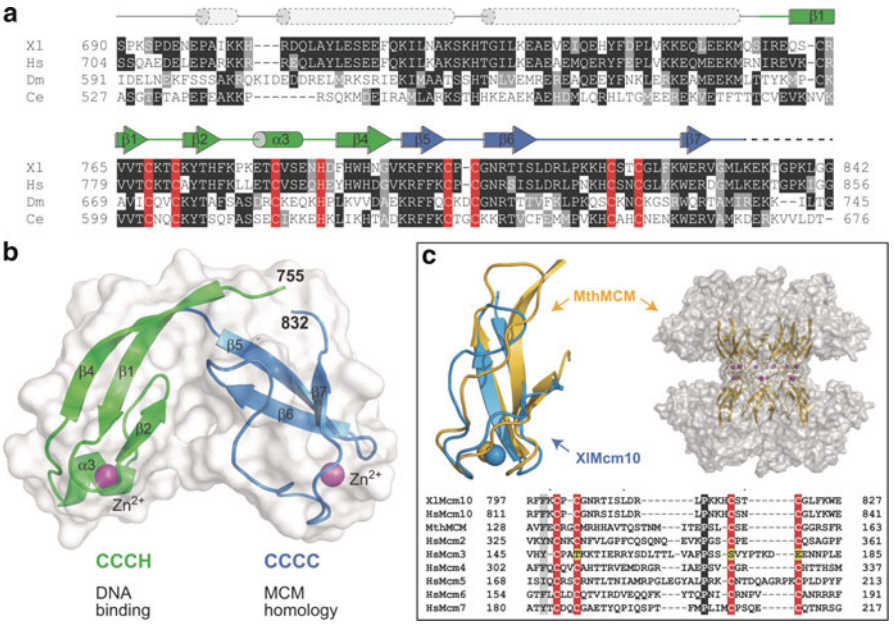


Fig. 11.4 NMR structure of xMcm10-CTD zinc binding region. (**a**) Sequence alignment of Mcm10-CTD. Secondary structure elements shown above the sequence are either predicted (*grey*) or determined from the NMR structure (*green*, *blue*). CCCH and CCCC zinc coordinating residues are highlighted in *red*. (**b**) The NMR structure of xMcm10 (756–842) is shown as a ribbon with a transparent *grey* molecular surface. (**c**) Structural and sequence alignment of the CCCC zinc ribbon from xMcm10 (aa 796–830) (*blue*) and MthMCM (*gold*, PDB ID 1LTL). In the crystal structure of the MthMCM N-terminal domain (top right of panel), the CCCC zinc ribbon mediates head-to-head double hexamer formation. The sequence alignment of this region (bottom of panel) shows the CCCC motif to be conserved in human Mcm2-7

in Mcm8 and Mcm9 proteins as well (Robertson et al. 2010). Mcm10 has been shown to interact directly with several subunits of Mcm2-7 helicase (Gambus et al. 2006; Izumi et al. 2000; Lee et al. 2003) and the recent finding that *Drosophila* Mcm10's interaction with Mcm2 is localized to the CTD (Apger et al. 2010) suggests that the CCCC zinc motif in both proteins may be the point of contact. The Mcm2-7 double hexamer that is loaded onto chromatin in the pre-RC (Remus et al. 2009) is able to separate during DNA unwinding (Yardimci et al. 2010), leading us to predict that if Mcm10-Mcm2-7 interactions are indeed facilitated by the zinc motifs, then this interaction would take place only after fork firing. Of course this is highly speculative and additional experiments are needed to define this aspect of Mcm10's function. What is known with certainty is that Mcm10 interacts with both the helicase and Pol α. Most likely, Mcm10 serves as a scaffold to co-localize the essential players within the replisome during the initiation and elongation phases of replication (Lee et al. 2010; Ricke and Bielinsky 2004; Robertson et al. 2010).

11.5 Implications of Modular Architecture for Function

Modular architecture is a common feature of DNA processing proteins that allows for the coordination of distinct biochemical activities (Stauffer and Chazin 2004). Flexible linkers between structured domains allow those domains to accommodate DNA and protein binding partners simultaneously, by virtue of the fact that the domains retain their structure while distance and angular adjustments are made between them. When tandem domains bind to the same entity, affinity for that entity is often increased relative to the strength of binding by one domain or the other. In addition, many DNA processing proteins contain bifunctional folds, which are known to bind both DNA and other proteins. Thus, when two different entities compete for the same binding site, it promotes molecular hand-off that facilitates the progression of the DNA processing pathway.

The structural organization of Mcm10 exemplifies each of these general features of DNA processing proteins. First, the attachment of the functional domains of Mcm10 by unstructured linkers, coupled with the spatial separation of protein and DNA binding sites, may allow it to bind both DNA and proteins simultaneously. Robertson et al. reported NMR spectra showing that the ID and the CTD of xMcm10 retain their individual structural properties in the context of a larger "ID + CTD" construct containing both domains and that the interdomain linker region is unstructured and flexible (Robertson et al. 2010). Second, the full-length xMcm10 protein, as well as the ID + CTD construct, binds DNA with 10-fold greater affinity than either the ID or CTD alone (Robertson et al. 2008; Warren et al. 2009), and ID + CTD binds Pol α p180 with 15-fold greater affinity than Mcm10-ID alone (Warren et al. 2009). Lastly, ssDNA and the N-terminal region of p180 compete for binding to the OB-fold cleft of Mcm10-ID (Warren et al. 2009). dsDNA also binds to essentially the same site on Mcm10-ID (Warren et al. 2008). Moreover, the PIP box predicted in the scMcm10 sequence (Ricke and Bielinsky 2006) coincides with the OB-fold β3 strand, suggesting that the OB-fold can bind to PCNA as well. Indeed, xMcm10 Phe324, which corresponds to the residue in scMcm10 that mediates interaction with PCNA (Das-Bradoo et al. 2006), had a modest effect on DNA binding (Warren et al. 2008).

The interaction of multiple binding partners with identical sites on Mcm10 fits with two distinct models of molecular hand-off (Fig. 11.5a, b). In the first, Mcm10-ID binds to ssDNA while the CTD is used to recruit a protein partner (e.g., Pol α p180). In the second model, the CTD binds ssDNA and ID recruits a protein binding partner via its OB-fold. Hand-off would be facilitated in either scenario by competition between the binding partner and Mcm10 for the exposed ssDNA or alternatively, for the OB-fold in Mcm10-ID. Depending on the oligomeric state of Mcm10 *in vivo*, there could be more than one subunit of the ID and the CTD present at the origin, increasing the number of possible interaction points and competition events (Fig. 11.5c). Future studies of the oligomerization state of Mcm10 and of the order of events at the origin will be needed to clarify which model is in play at each stage of replication.

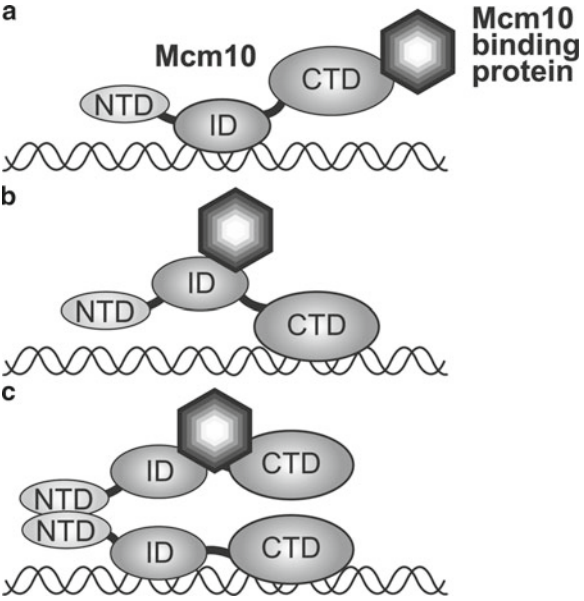


Fig. 11.5 Three possible models for Mcm10 hand-off of other proteins (e.g., Pol α) onto DNA. (**a**, **b**) Either Mcm10-ID or CTD interact with DNA, while the non-DNA bound domain is free to bind protein cargo. (**c**) ID + CTD together create a high-affinity DNA binding platform, and Mcm10 self-association through the NTD would present an additional free binding platform to localize proteins to the DNA

Its modular architecture and lack of enzymatic activity suggest that Mcm10 serves as a scaffold for the coupling of protein and DNA interactions during replication initiation. For example, a head-to-head Mcm10 dimer could couple events on the leading and lagging strands, or physically tether the helicase and Pol α, while retaining the polarity necessary for fork progression. Interestingly, recent studies have shown that yeast Mcm10 displays differential packing on ssDNA versus dsDNA (Eisenberg et al. 2009), suggestive of an Mcm10-DNA scaffold during origin melting or helicase unwinding. The authors speculated that a change in Mcm10 conformation or oligomeric state could facilitate strand separation.

11.6 Summary and Future Perspectives

Despite decades of work, Mcm10 remains an essential yet mysterious player in DNA replication. As one of the first proteins to load after pre-RC formation, Mcm10 is needed for subsequent protein loading and downstream events in DNA replication initiation. Mcm10 interacts with multiple replisome components and DNA. Its interactions with ssDNA and Pol α are mediated by the conserved ID and CTD through OB-fold and zinc finger structural elements. Crystal and NMR structures have elucidated the details of the ID-DNA interactions and have begun to address the binding activity within the CTD. Full-length xMcm10 forms a number of oligomeric species, which may be assembled through coiled coil interactions within the NTD. The functional significance of Mcm10's self-assembly and its interactions

within the replisome, the structure of the NTD, the mechanisms of multi-domain DNA binding activities, and the effects of ubiquitylation and other post-translational modifications on Mcm10 structure and function are all questions that remain unanswered.

The nature of Mcm10 self-assembly is critical for understanding its role at the replication fork, although the structural and functional relationship between the apparent multiple oligomeric states is not at all clear from the literature. A dimerization model best explains the physical and genetic evidence for Mcm10's interaction with both leading and lagging strand polymerases at a replication fork (Fien et al. 2004; Ricke and Bielinsky 2006; Robertson et al. 2008), but this remains to be determined. Detailed structural analyses of the N-terminal domain may help to address this issue. In addition, studies of the configuration(s) of the tandem ID and CTD in complex with DNA, Pol α and other protein binding partners will yield insight into the mechanisms by which Mcm10 acts as a scaffold at the replication origin.

A growing body of research also suggests Mcm10 plays a role in elongation. The first glimpse of a potential role for Mcm10 in fork progression came from the observation in *S. cerevisiae* that Mcm10 mutants delayed completion of DNA synthesis after cells were released from hydroxyurea arrest (Kawasaki et al. 2000). Mcm10 interacted genetically with Pol δ and Pol ε (Kawasaki et al. 2000) and a physical interaction with replisome progression complexes, which exist at DNA replication forks, has been observed in yeast (Gambus et al. 2006). In addition, a diubiquitylated form of scMcm10 interacts with PCNA, suggesting that Mcm10 directly participates in DNA elongation (Das-Bradoo et al. 2006). Pacek et al. showed that Mcm10 travels with the replication fork by inducing specific replication fork pausing on biotin-streptavidin-modified plasmids in *Xenopus* egg extracts (Pacek et al. 2006). In these experiments, Mcm10 was found to localize to the vertebrate DNA replication fork by chromatin immunoprecipitation. Finally, recent work in yeast suggested that Mcm10 coordinates the activities of the Mcm2-7 helicase and Pol α and ensures their physical stability at the elongating replication fork (Lee et al. 2010).

Although the majority of work to date has been focused on its role in DNA replication, Mcm10 has also been shown to be important for transcriptional gene silencing (Apger et al. 2010; Douglas et al. 2005; Liachko and Tye 2005, 2009). scMcm10 physically interacts with Sir2 and Sir3, two essential silencing factors in *S. cerevisiae* (Douglas et al. 2005). Moreover, Mcm10 mediates interactions between Sir2 and subunits 3 and 7 of the Mcm2-7 helicase via a ~100-residue segment at its C-terminus. Mutations to this region of Mcm10 caused silencing defects, but had no detrimental effect on replication (Liachko and Tye 2009). The corresponding segment in the *Xenopus* protein resides within an unstructured linker between the ID and CTD (Fig. 11.1b), which suggests that either the yeast protein has an organism-specific function or the vertebrate Mcm10s have an as yet uncharacterized role in gene silencing. The yeast sequence between residues 515 and 523 is predicted to be an amphipathic helix (Liachko and Tye 2009), a finding which warrants further investigation into the analogous segments of its orthologs.

In summary, Mcm10 lies at the heart of the replication initiation pathway. It loads early onto licensed replication origins and is necessary for pre-RC activation and

origin melting as a result of its interactions with DNA and many of the enzymes involved in fork progression. In addition to its essential role in establishing active replication forks at the origin, Mcm10 is involved in other aspects of genome utilization. The structures and interactions between Mcm10 and its binding partners are adding to a growing body of knowledge for how multi-conformation scaffolding proteins are used to maintain the integrity of the genome. To further enhance our understanding of the mechanisms involved in replisome assembly and function, the next step is to utilize the existing structures of the Mcm10 DNA binding domains as a foundation to build up larger sub-complexes, taking advantage of the extensive network of Mcm10 interactions. This higher resolution picture of the replisome will be critical to understand the transactions involved at the replication fork, including DNA synthesis, damage response and repair, and cell cycle regulation.

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